Changes in Gene Expression during Tomato Fruit Ripening[1]

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ABSTRACT

Total proteins from pericarp tissue of different chronological ages from normally ripening tomato (*Lycopersicon esculentum* Mill. cv Rutgers) fruits and from fruits of the isogenic ripening-impaired mutants *rin*, *nor*, and *Nr* were extracted and separated by sodium dodecylsulfate-polyacrylamide gel electrophoresis. Analysis of the stained bands revealed increases in 5 polypeptides (94, 44, 34, 20, and 12 kilodaltons), decreases in 12 polypeptides (106, 98, 88, 76, 64, 52, 48, 45, 36, 28, 25, and 15 kilodaltons), and fluctuations in 5 polypeptides (85, 60, 26, 21, and 16 kilodaltons) as normal ripening proceeded. Several polypeptides present in ripening normal pericarp exhibited very low or undetectable levels in developing mutant pericarp. Total RNAs extracted from various stages of Rutgers pericarp and from 60 to 65 days old *rin*, *nor*, and *Nr* pericarp were fractionated into poly(A)$^+$ and poly(A)$^-$ RNAs. Peak levels of total RNA, poly(A)$^+$ RNA, and poly(A)$^+$ RNA as percent of total RNA occurred between the mature green to breaker stages of normal pericarp. *In vitro* translation of poly(A)$^+$ RNAs from normal pericarp in rabbit reticulocyte lysates revealed increases in mRNAs for 9 polypeptides (116, 89, 70, 42, 38, 33, 31, 29, and 26 kilodaltons), decreases in mRNAs for 2 polypeptides (41 and 35 kilodaltons), and fluctuations in mRNAs for 5 polypeptides (156, 53, 39, 30, and 14 kilodaltons) during normal ripening. Analysis of two-dimensional separation of *in vitro* translated polypeptides from poly(A)$^+$ RNAs isolated from different developmental stages revealed even more extensive changes in mRNA populations during ripening. In addition, a polygalacturonase precursor (54 kilodaltons) was immunoprecipitated from breaker, turning, red ripe, and 65 days old *Nr* *in vitro* translation products.

Ripening of climacteric fruits is a dynamic transitional period during which many easily perceived changes, such as alterations in pigmentation, firmness, aroma, sweetness, and acidity, take place. Stimulation of protein synthesis appears to be an essential component of the ripening process as judged by incorporation of radioactive amino acids and inhibition of ripening following treatment with protein synthesis inhibitors (8). The activities of several enzymes change during ripening of climacteric fruits (14, 23) including tomato PG,[2] which is synthesized *de novo* and accumulates as ripening proceeds (5, 14, 21, 29). In addition, changes in the levels of specific mRNAs during tomato and

avocado fruit ripening also have been shown (6, 7, 10, 17, 22, 26, 27). Patterns of tomato polypeptides translated *in vitro* in response to mRNAs from early stages of fruit development differ from those translated in response to mRNAs from later stages of fruit development (10, 22, 26, 27). Several tomato fruit mRNAs recently have been cloned (17, 26). However, a controversy currently exists as to the precursor size of tomato fruit PG as translated *in vitro* from isolated mRNAs (10, 24-26).

We have been investigating changes in the populations of proteins and mRNAs during normal tomato fruit development and the extent to which these changes differ in fruit from three abnormally ripening mutants, namely *rin*, *nor*, and *Nr*. Preliminary results have been presented previously (4, 11). Our results show that the relative predominance of many proteins changes during tomato fruit development and that several proteins which accumulate during normal tomato fruit ripening either fail to do so or do so at reduced levels in the three ripening mutants investigated. Analysis of mRNA populations (based upon *in vitro* translation of poly(A)$^+$ RNAs from different stages of fruit development) from normal and mutant phenotypes indicates even more extensive changes in gene expression during the ripening process. In addition, our results indicate that the mol wt of tomato PG precursor as translated *in vitro* in a rabbit reticulocyte lysate system from poly(A)$^+$ RNAs is 54,000.

MATERIALS AND METHODS

Plant Material. Tomato plants (*Lycopersicon esculentum* Mill.) of the normally ripening cv Rutgers and of nearly isogenic lines of the *rin*, *nor*, and *Nr* ripening-impaired mutants (eight, four, and six backcrosses to Rutgers, respectively) were grown in a soil mixture (20% topsoil, 40% sphagnum moss, 40% perlite) in pots (25 cm diameter, 10 L) under a 24°C (day) to 18°C (night) greenhouse environment. Photoperiod consisted either of 14 to 15 h sunlight during LD months or 14 h supplemental light from high intensity discharge lamps during SD months. Fruit set was accomplished by hand pollination and limited to four or less per inflorescence. Fruits were harvested at appropriate times, inner contents excised, and the pericarp tissue frozen immediately by immersion in liquid N_2. Pericarp tissues were stored at −80°C until use.

Electrophoresis and Protein Analysis. Pericarp used for protein analysis was lyophilized and ground to a fine powder. Three-mg samples of pericarp powder were suspended in 0.1 ml of sample buffer (Laemmli's buffer [16] supplemented with 1 mM phenylmethylsulfonyl fluoride and 2.5 mM Na_2EDTA), placed in a boiling H_2O bath for 3 min, cooled and spun in an Eppendorf 5412 microcentrifuge for 3 min. Supernatants (30 μl) were electrophoresed (16) in slab gels (0.75 × 120 × 140 mm) at 12.5 mamp per gel with gels poured as 10 to 16% (w/v) acrylamide gradients containing 1% SDS. Gels were fixed and stained with 50% (v/v) methanol, 12.5% (v/v) acetic acid containing 0.025% (w/v) Coomassie blue R-250, destained twice in 40% (v/v) methanol, 10% (v/v) acetic acid, then once in 5% (v/v) methanol, 7% (v/v) acetic acid. Gels were scanned densitometrically at 550

[2] Abbreviations: PG, polygalacturonase (EC 3.2.1.15; PG1 and PG2 are high and low mol wt forms, respectively); *rin*, *nor*, and *Nr*, ripening inhibitor, nonripening, and never ripe tomato mutants; PE, pectinesterase (EC 3.2.1.11); P-200 or P-100, polyacrylamide 200 kD or 100 kD exclusion gel filtration; IgG, immunoglobulin G; IMG, MG, Br, Tu, Ri, and RR, immature green, mature green, breaker, turning, ripe, and red ripe stages of tomato fruit development; gFW, gram fresh weight; pI, the pH value of the isoelectric point of a given substance.

nm with a Beckman DU-8 spectrophotometer. Percent areas for individual peaks within a given scan were computed using a baseline value obtained from the lowest absorbance reading of that scan.

Table I. *Ages of Tomato Fruit Developmental Stages Used for Analysis of Proteins Present in Pericarp Tissue*

Rutgers (stage)	Genotype			Gel Lane in Figure 1
	rin	nor	Nr	
	d after pollination			
30 (IMG)	30	30	30	2
43 (MG)	46	46	46	3
49 (Br)	50	50	54	4
53 (Tu)	60	60	60	5
58 (Ri)	70	70	75	6
70 (RR)	85	80	85	7
	100	100	95	8
	120	120	115	9

In vitro translated polypeptides were analyzed as described above except equal amounts of TCA-precipitable L-[^{35}S]Methionine proteins were applied to each lane. Gels were equilibrated in 7% (v/v) acetic acid, impregnated for 30 min with Fluoro-Hance (Research Products International Corp., Mount Prospect, IL), vacuum-dried onto Whatman 3MM filter papers, and exposed to Kodak XAR-5 film at −80°C. Fluorograms were scanned densitometrically with a Beckman DU-8 spectrophotometer using white light. Two-dimensional analyses of *in vitro* translated polypeptides were accomplished as described (19) except that samples were focused at 1 W per tube gel until a stable current was achieved.

Standard protein markers from Pharmacia were as follows: phosphorylase b, 94.0 kD; BSA, 67.0 kD; ovalbumin, 43.0 kD, carbonic anhydrase, 30.0 kD; soybean trypsin inhibitor, 20.1 kD; α-lactalbumin, 14.4 kD. PG2 (45 and 44 kD molecular forms) and PE (34 kD) standards were purified to apparent homogeneity as described below. Ampholytes of pH 3 to 10 were from Isolab, Inc. (Akron, OH).

RNA Extraction. To extract total RNA, 100 g of frozen tomato pericarp tissue was powdered under liquid N_2 and stirred for 30
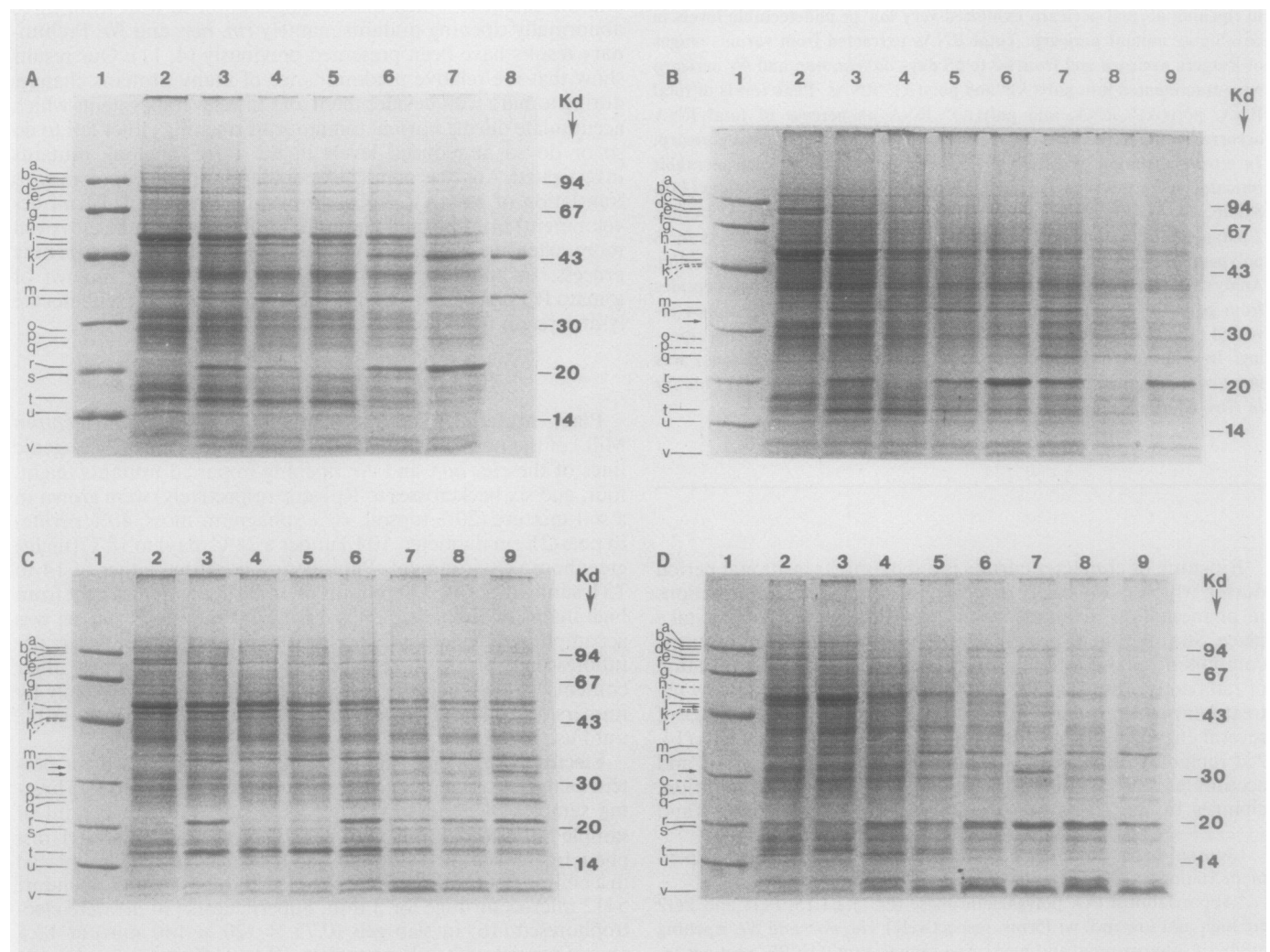

FIG. 1. SDS-PAGE of total polypeptides extracted from various chronological ages of normal and mutant tomato pericarp. Mol wt standards are shown in lane 1; other lane numbers represent samples from various ages as shown in Table I. Letters correspond to polypeptides quantified in Table II. Broken lines indicate polypeptides that are either reduced or undetectable in mutant genotypes. Arrows show polypeptides present in mutants that are not evident in normal pericarp. The electrophoretic mobilities of purified PG2 (45 and 44 kD molecular forms) and PE (34 kD) are shown in lane 8 of panel A. A, Rutgers; B, *rin*; C, *nor*; D, *Nr*.
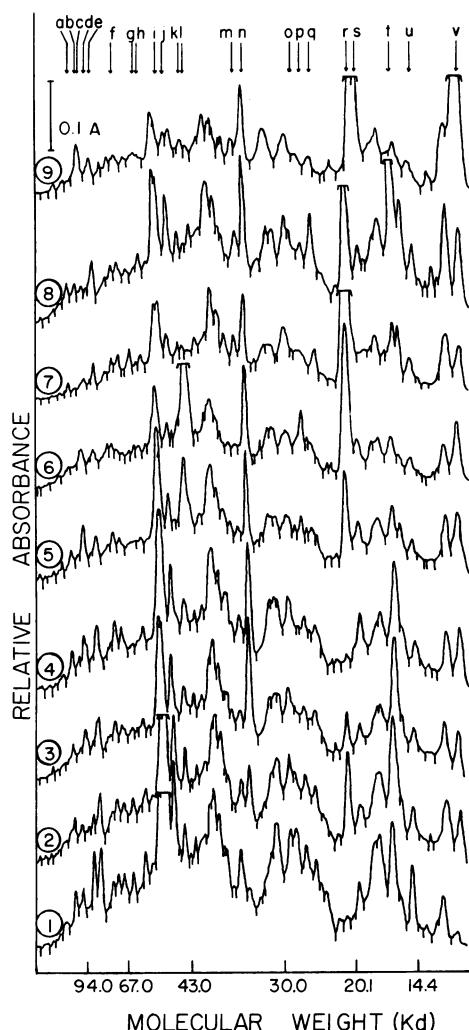
FIG. 2. Densitometric scans at 550 nm of selected SDS-PAGE gel lanes from Figure 1. Letters represent peaks characterized in Table II. Scans are as follows: 1, IMG; 2, MG; 3, Br; 4, Tu; 5, Ri and 6, RR of Rutgers; 7, *rin* 70 d; 8, *nor* 70 d; 9, *Nr* 75 d.

min in 200 ml of lysis medium (9) containing 1% (v/v) β-mercaptoethanol and 10 mM ribonucleoside vanadyl complexes (3). The aqueous phase was deproteinized by extraction twice with 100 ml of phenol solution (9):chloroform:isoamyl alcohol (50:48:2, v:v:v) with centrifugations at 10,000g for 5 min. Nucleic acids were precipitated with two volumes of ethanol plus 0.05 volume of 4 M sodium acetate (pH 6.0) at −20°C for at least 3 h then centrifuged at 16,000g for 15 min. The precipitate was resuspended in 40 ml ice-cold H_2O using a Dounce homogenizer and clarified by centrifugation at 12,000g for 5 min. The cycle of precipitation and resuspension was repeated before RNA was precipitated in 3 M sodium acetate (pH 6.0) for 1.5 h at 0°C. The precipitate was collected by centrifugation at 27,000g for 20 min and the pellet resuspended in 5 ml H_2O. The preceding step was repeated once more and the redissolved RNA precipitated with 2 volumes of ethanol plus 0.05 volume of 4 M sodium acetate (pH 6.0) at −20°C for at least 3 h. The RNA pellet obtained by centrifugation at 12,000g for 15 min was dried under vacuum until slightly moist and solubilized in 5 ml H_2O. A small aliquot of RNA was diluted and scanned with a Beckman DU-8 spectrophotometer from 220 to 320 nm. All RNA preparations showed an $A_{260}:A_{280}$ ratio of at least 2 with negligible absorbance above A_{310}. RNA content was calculated assuming an extinction coef-

ficient of 25.0 A_{260} units mg^{-1} RNA. Poly(A)$^+$ RNAs were separated using oligo (dT)-cellulose chromatography (1) except that the first elution buffer was omitted. RNA was subjected to three passages through an oligo (dT)-cellulose column before elution; the first two passages were preceded by heating the RNA sample to 65°C for 5 min followed by rapid cooling on ice before each passage at room temperature. Fractions containing poly(A)$^+$ RNAs were pooled and quantitated for RNA content based upon A_{260}. RNA was precipitated with 0.0375 volume of 4 M sodium acetate (pH 6.0) plus 1.5 volumes of ethanol at −20°C for at least 4 h and the precipitate was collected by centrifugation at 243,500g (Beckman L5-65 ultracentrifuge, Ti-40 rotor) for 2 h at 4°C. The RNA pellet was dried under vacuum, resuspended in 50 μl H_2O, spun in an Eppendorf microcentrifuge for 2 min and the supernatant stored as aliquots at −80°C. The integrity of poly(A)$^+$ RNAs was verified relative to poly(A)$^-$ RNAs by electrophoresis in native and denaturing agarose gels (data not shown).

In Vitro Translation and Analysis of L-[^{35}S]Methionine Incorporation. Preparation of rabbit reticulocyte lysates and conditions for cell-free translations were as described (20) except that DTT was added to a final concentration of 0.8 mM for translations. Four μg each of poly(A)$^+$ RNAs were translated in 60 μl reaction mixtures for 90 min at 30°C using 10 μCi of L-[^{35}S]methionine (New England Nuclear; 1022 Ci $mmol^{-1}$). Duplicate 2 μl aliquots from each sample were removed following incubation and the remainder frozen at −80°C. Aliquots were added to 1 ml of icecold 10% (w/v) TCA containing 5 mM unlabeled methionine and 150 μg BSA. Precipitates in suspension were placed in a boiling H_2O bath for 10 min, cooled in ice H_2O, and collected on glass fiber disks by filtration. Precipitates were washed (at 0°C) three times with a total of 10 ml of 10% (w/v) TCA containing 5 mM methionine and once with 3 ml of 95% (v/v) ethanol, dried and counted in a Beckman LS 6800 spectrometer.

Enzyme Extractions. PE, PG1, and PG2 were extracted from red ripe, turning, and red ripe tomato pericarp, respectively, essentially as described for PG (12). PG1 was purified further by sequential chromatography over P-200 and carboxymethyl-cellulose columns. PE and PG2 were purified to apparent homogeneity (as analyzed by single dimension SDS-PAGE) by sequential chromatography over DEAE-cellulose, carboxymethyl-Sephadex, and P-100 columns. PG and PE were assayed for activity as described (12, 30).

Preparation of PG Antibody. Three New Zealand White rabbits were injected subcutaneously on two occasions with emulsions of purified PG2 in Freund's complete adjuvant followed periodically by booster injections of emulsions of purified PG2 in Freund's incomplete adjuvant. Sera obtained from bleedings were pooled, clarified by centrifugation at 3,000g then 20,000g for 10 min each and total IgG extracted by precipitation in 1.9 M $(NH_4)_2SO_4$. The pellet collected at 10,000g for 20 min was washed with 1.9 M $(NH_4)_2SO_4$, centrifuged as before, and redissolved in H_2O. The IgG solution was diluted to the original volume of pooled sera and tested for reactivity against PG1 and PG2 by Ouchterlony double immunodiffusion. Medium for immunodiffusion and detection of precipitin lines were as described (31).

Immunoprecipitation. A PG precursor from total *in vitro* translation products was immunoprecipitated (15) using polyclonal PG2 antibodies and Pansorbin (Behring Diagnostics, La Jolla, CA). Equal TCA-precipitable cpm of [^{35}S]methionine were aliquoted after *in vitro* translations for immunoprecipitation. Immune complexes were resuspended in 20 μl of electrophoresis sample buffer, placed in a boiling H_2O bath for 5 min, and centrifuged for 3 min in an Eppendorf microcentrifuge. Supernatants were removed and the pellets reextracted with 20 μl of the same buffer. The two supernatants from each sample were pooled. Ten μl from each sample were counted to determine

Table II. *Changes in the Levels of Major Polypeptides during Development of Normal and Mutant Tomato Fruit Pericarp*

Numbers indicate the percent area a particular peak represents from the total area of a given scan from Figure 2. Letters represent either bands from Figure 1 or corresponding peaks from Figure 2. Dashes indicate the absence of a discrete band at that particular position on the gel from Figure 1.

Polypeptides		Fruit Genotype and Age (d)								
		Rutgers						rin	nor	Nr
Band or peak	Mol mass	30	43	49	53	58	70	70	70	75
	kD					% area				
a	106	1.2	1.0	1.0	1.2	1.0	—	0.3	0.6	0.7
b	98	1.3	0.8	0.8	0.8	—	—	—	0.7	—
c	94	—	—	1.2	1.7	1.7	1.7	—	0.8	1.9
d	88	1.5	0.9	0.7	0.7	—	—	—	—	—
e	85	2.1	1.7	1.5	2.0	1.3	1.5	0.8	1.4	1.4
f	76	1.0	1.0	1.3	1.2	—	—	—	—	—
g	64	1.6	1.9	—	—	—	—	0.9	—	—
h	60	1.9	1.5	1.9	1.3	1.3	1.6	—	1.2	—
i	52	8.9	4.7	3.0	4.1	2.2	—	2.8	2.8	1.4
j	48	3.9	3.7	3.3	3.0	2.5	2.3	2.9	3.1	2.0
k	45	2.7	2.9	3.0	3.0	—	—	—	2.0	—
l	44	—	—	—	—	6.2	7.9	—	—	1.3
m	36	2.8	2.6	2.2	2.4	1.8	—	2.3	2.7	2.5
n	34	1.5	2.9	5.1	4.0	4.2	4.9	5.1	4.0	5.0
o	28	3.3	2.5	—	—	—	—	—	2.7	—
p	26	3.3	2.9	2.0	2.0	1.9	3.8	—	1.8	—
q	25	2.1	2.0	2.5	1.9	1.8	—	1.8	2.6	1.4
r	21	—	3.3	2.3	—	4.0	8.1	14.0	4.9	5.5
s	20	1.4	1.9	1.9	2.3	2.2	2.5	—	1.9	5.5
t	16	3.5	5.0	5.1	4.6	3.4	2.8	3.1	6.3	—
u	15	1.8	1.8	1.5	1.6	1.5	1.0	1.5	1.7	—
v	12	0.3	1.3	1.1	2.4	3.2	3.0	2.4	2.6	12.1

Table III. *Yields and Translatability of RNA Isolated from Normal and Mutant Tomato Pericarp*

Genotype	Stage of Development	RNA		Poly(A)$^+$ RNA	[^{35}S] Methionine Incorporated ng^{-1} poly(A)$^+$ RNA 90 min^{-1}
		Total	Poly(A)$^+$		
		μg/gFW		% of total RNA	cpm
Rutgers	IMG	77.43	0.63	0.81	856
	MG	91.20	1.44	1.58	627
	Br	83.55	1.55	1.85	840
	Tu	65.08	1.06	1.63	684
	RR	29.75	0.43	1.45	904
rin	65 d	83.36	0.72	0.87	1336
nor	60 d	99.56	0.64	0.64	1126
Nr	65 d	35.86	0.21	0.60	854

radioactivity and the remaining portions subjected to SDS-PAGE.

RESULTS

Changes in Polypeptide Populations during Normal Tomato Pericarp Development. Total proteins extracted from normally ripening 'Rutgers' pericarp of various ages (Table I) were subjected to SDS-PAGE (Fig. 1A). While the levels of many polypeptides remained relatively constant during pericarp development, many others exhibited notable increases, decreases, or fluctuations during this period (Figs. 1A, 2). Increases in five

polypeptides (94, 44, 34, 20, and 12 kD) were observed as development proceeded (Table II). Of these, the 20 and 12 kD polypeptides steadily increased throughout development. The 94 and 44 kD polypeptides appeared at Br and Ri stages, respectively, while the 34 kD polypeptide, although present at all stages sampled, exhibited a higher level throughout ripening. The electrophoretic mobilities of the 44 and 34 kD polypeptides were similar to those of purified PG2 and PE, respectively (Fig. 1A). Although other polypeptides of similar mol wt may contribute to the staining intensity in these two regions of the gel, taken together these two bands constitute almost 13% of the total extractable protein by the RR stage (Table II).
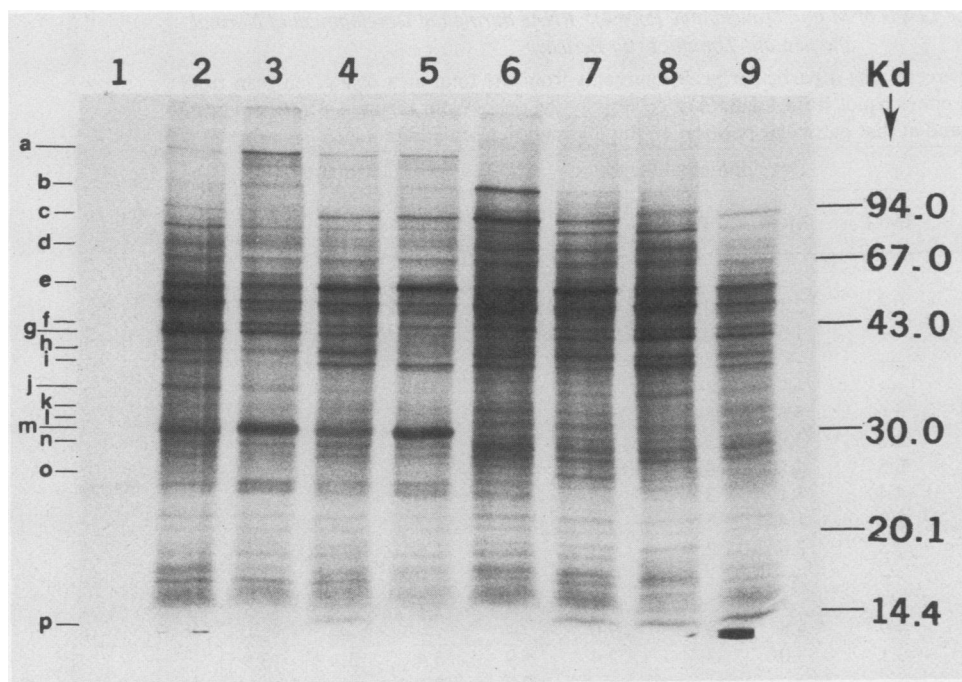
FIG. 3. Fluorogram of SDS-PAGE of [^{35}S]methionine polypeptides translated *in vitro* from tomato pericarp poly(A)$^+$ RNAs. Equal TCA-precipitable counts (1 × 10^5 cpm) were applied to each lane except lane 1 (5 × 10^3 cpm). Lanes are as follows: 1, no added poly(A)$^+$ RNA; 2, IMG; 3, MG; 4, Br; 5, Tu; 6, RR; 7, *rin* 65 d; 8, *nor* 60 d; 9, *Nr* 65 d. Letters represent polypeptides whose poly(A)$^+$ RNA levels change at or beyond the MG stage in normal Rutgers.
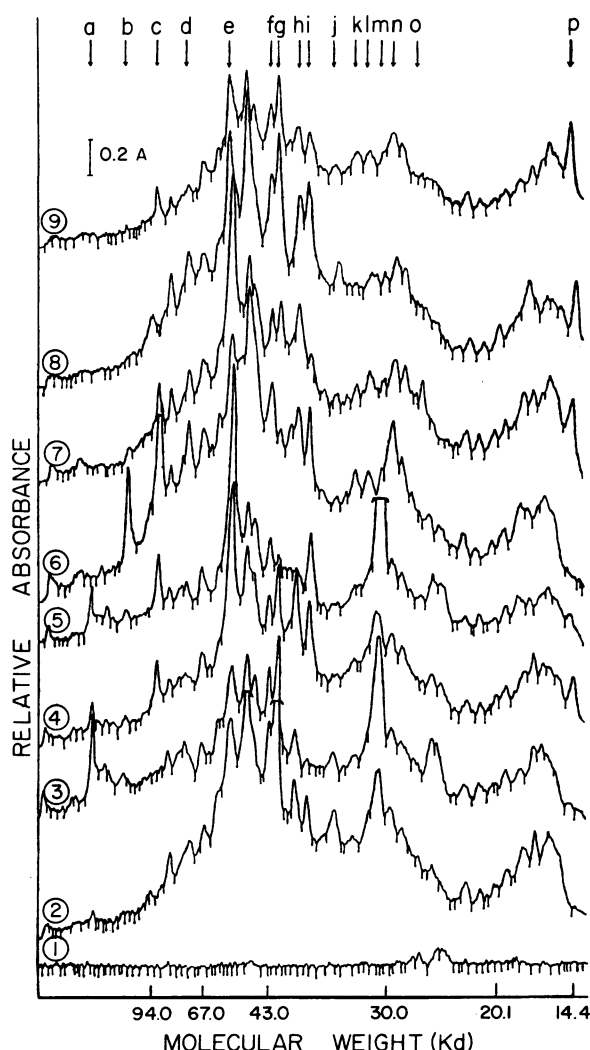

FIG. 4. Densitometric scans of the fluorogram of *in vitro* translated polypeptides. Numbers of individual scans correspond to gel lanes from Figure 3. Letters represent peaks characterized in Table IV.

Twelve polypeptides (106, 98, 88, 76, 64, 52, 48, 45, 36, 28, 25, and 15 kD) decreased during the development of Rutgers pericarp. Of these, the 64 and 28 kD polypeptides were present only during the IMG and MG stages, the 52 kD polypeptide dramatically declined throughout development while the 48, 36, and 15 kD polypeptides exhibited more steady declines. The remaining six polypeptides (106, 98, 88, 76, 45, and 25 kD) achieved relatively stable levels for at least three consecutive stages before finally disappearing at fully ripe stages. Five polypeptides (85, 60, 26, 21, and 16 kD) showed fluctuations during pericarp development, especially the 21 kD polypeptide, which displayed high levels at the MG, Br, Ri, and RR stages but was virtually absent at the IMG and Tu stages.

Comparison between Normal *versus* Mutant *rin*, *nor*, and *Nr* Polypeptides during Pericarp Development. Several polypeptides present in normal pericarp were either reduced or undetectable in one or more mutant tissues (Fig. 1). The *rin* mutant exhibited this trait for the 45, 44, 28, 26, and 20 kD polypeptides, *nor* for the 44 kD polypeptide, and *Nr* for the 60, 45, 44, 28, and 26 kD polypeptides. The 94 kD polypeptide was present very early in *rin* tissue as compared to normal but declined rapidly thereafter. All three mutants contained relatively high levels of a 25 kD polypeptide, especially during later tissue ages (Fig. 1). Certain polypeptides were present in 75 d or older mutant pericarp which were not detectable at any stage of normal tissue. These were 33 kd in *nor*, 32 kD in *rin* and *nor*, and 31 and 47 kD in *Nr* (Fig. 1).

The disappearance of high mol wt polypeptides and increase in low mol wt polypeptides occurred by roughly 70 d after pollination for all four genotypes, especially for *rin* and *Nr*, suggesting that certain senescent and/or proteolytic effects were exerted in a chronological fashion in mutant pericarp even in the absence of normal ripening. However, pericarp from the three mutants seems to contain many polypeptides that usually occur only during early stages of normal pericarp development.

Quantification of Total and Poly(A)$^+$ RNA Levels during Normal and Mutant Pericarp Development. Total RNA from normal pericarp reached a maximum at the MG stage (about 91 µg/gFW) then steadily declined during ripening to about one-third that amount at the RR stage (Table III). Poly(A)$^+$ RNA levels peaked at Br (1.6 µg/gFW), and then declined to about one-fourth of that level by the RR stage. However, poly(A)$^+$ RNA as a percent of total RNA, which was highest at the Br

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Table IV. *Changes in the Levels of Major Translatable Poly(A)⁺ RNAs during the Development of Normal and Mutant Tomato Fruit Pericarp*

Numbers indicate the percent area a particular peak represents from the total area of a given scan from Figure 4. Letters represent either bands from Figure 3 or corresponding peaks from Figure 4. Dashes indicate the absence of a discrete band at that particular position on the fluorogram from Figure 3.

Polypeptides		Developmental Stages					Mutants		
Band or peak	Mol mass	IMG	MG	Br	Tu	RR	rin	nor	Nr
	kD					% area			
a	156	0.3	2.1	0.8	1.2	—	—	—	—
b	116	—	1.5	0.7	1.2	2.3	0.7	—	—
c	89	—	—	2.4	2.6	4.5	1.3	—	0.7
d	70	—	—	—	1.6	3.7	2.4	3.0	—
e	53	6.6	5.6	8.7	11.3	6.3	8.8	7.3	5.2
f	42	3.3	3.8	3.1	3.3	5.0	3.8	4.5	4.2
g	41	6.5	4.5	4.0	2.0	2.1	3.6	5.8	4.6
h	39	4.4	3.1	3.8	2.8	3.7	4.0	3.8	4.2
i	38	2.9	1.4	3.3	3.4	3.1	1.7	2.4	3.8
j	35	2.8	2.8	2.4	1.7	1.6	1.5	2.1	2.7
k	33	—	—	—	—	2.8	1.9	—	—
l	31	—	—	—	—	3.1	4.2	3.6	3.4
m	30	4.9	6.8	5.5	9.0	—	—	—	—
n	29	3.1	3.0	3.3	2.4	5.9	3.4	3.1	3.9
o	26	—	—	—	—	0.7	3.1	—	—
p	14	—	—	1.7	—	—	2.1	1.9	3.1

stage, did not change appreciably during ripening. In addition, the *in vitro* translatability of poly(A)⁺ from various stages, as judged by [³⁵S]methionine incorporation, remained relatively unchanged.

Total RNA levels isolated from 60 to 65 d old *rin* and *nor* pericarp (about 83 and 100 μg/gFW, respectively) were similar to those of MG and Br stages of normal fruit. However, levels of poly(A)⁺ RNA for these mutants were relatively low (0.6–0.7 μg/gFW), displaying values similar to those characteristic of either RR or IMG stages of normal pericarp (0.4 and 0.6 μg/gFW, respectively). For *Nr* pericarp, the level of total RNA was similar to the normal RR stage, but the poly(A)⁺ RNA level was lower than that of any normal pericarp stage. On the other hand, percent poly(A)⁺ RNA values for all three mutants (0.6–0.8%) were similar to normal IMG but not RR values (0.8% *versus* 1.5%, respectively). The ability of poly(A)⁺ RNAs isolated from these mutants to incorporate[³⁵S]methionine into polypeptides was similar to or slightly higher than that of poly(A)⁺ RNAs from normal fruit (Table III).

Changes in mRNA Populations during Normal Pericarp Development. Changes in populations of poly(A)⁺ RNAs during ripening of normal fruit were characterized based upon their ability to translate polypeptides in micrococcal nuclease-treated rabbit reticulocyte lysate *in vitro* systems. Analysis of *in vitro* translation products separated by SDS-PAGE suggested increases in nine polypeptides (116, 89, 70, 42, 38, 33, 31, 29, and 26 kD), decreases in two polypeptides (41 and 35 kD), and fluctuations in five polypeptides (156, 53, 39, 30, and 14 kD) during development (Figs. 3, 4; Table IV). The polypeptides that increased during development consisted of six (116, 89, 70, 33, 31, and 26 kD) that appeared during or after the MG stage while three (42, 38, and 29 kD) appeared throughout development but increased by the RR stage. The 41 and 35 kD polypeptides decreased steadily from the IMG through RR stages. Polypeptides that fluctuated during development consisted of three types: one (14 kD) appeared only at the Br stage, two (53 and 39 kD) varied throughout development, and two others (156 and 30 kD) likewise varied but then disappeared at the RR stage. Two-dimensional analysis of labeled polypeptides translated from MG and

Tu poly(A)⁺ RNAs (Fig. 5, A and B) revealed even more extensive changes than those cited above.

Comparison of Normal *versus* Mutant *rin, nor,* and *Nr* mRNA Populations During Pericarp Development. Polypeptides translated by 60 to 65 d old *rin, nor,* and *Nr* poly(A)⁺ RNAs were somewhat mixed in their pattern when compared to Rutgers *in vitro* translation products (Figs. 3, 4; Table IV). Many polypeptides translated from the three mutant poly(A)⁺ RNAs also were translated from normal 70 d old RR pericarp poly(A)⁺ RNAs. These included the 53, 42, 41, 39, 38, 35, 31, and 29 kD polypeptides. The absence of the 156 and 30 kD polypeptides from 60 to 65 d old mutant profiles was consistent with the profile derived from RR poly(A)⁺ RNAs. However, several polypeptides (116, 89, 70, 33, 26, and 14 kD) displayed different patterns in at least one of the three mutants.

Immunoprecipitation of a PG Precursor. Polyclonal antibodies raised against the native form of purified PG2 showed a single precipitin line when challenged with PG2, PG1, or proteins extracted from ripening pericarp (Fig. 6). When these antibodies were employed in immunoprecipitation studies of *in vitro* translation products to determine the presence of a PG precursor, a 54 kD polypeptide was immunoprecipitated from Tu and RR stages (Fig. 7). This polypeptide also was present in smaller quantity at Br stage and in trace amount in 65 d old *Nr* pericarp. The broadness of the polypeptide band was due to its coinciding with the heterogeneous migration distance of the denatured nonradioactive heavy chains of total IgG used in the immunoprecipitation process. The average mol wt of the precursor band (54 kD) is approximately 10 kD larger than the apparent mol wt of mature PG2 (Fig. 1A).

DISCUSSION

Several investigations have been undertaken to gain an understanding of protein changes and molecular mechanisms underlying the regulation of tomato fruit ripening (2, 5, 10, 13, 14, 17, 21, 22, 24–27, 29, 30). However, a quantitative survey of total protein changes during various stages of normal tomato pericarp development and a comparison to those present in the *rin, nor,*
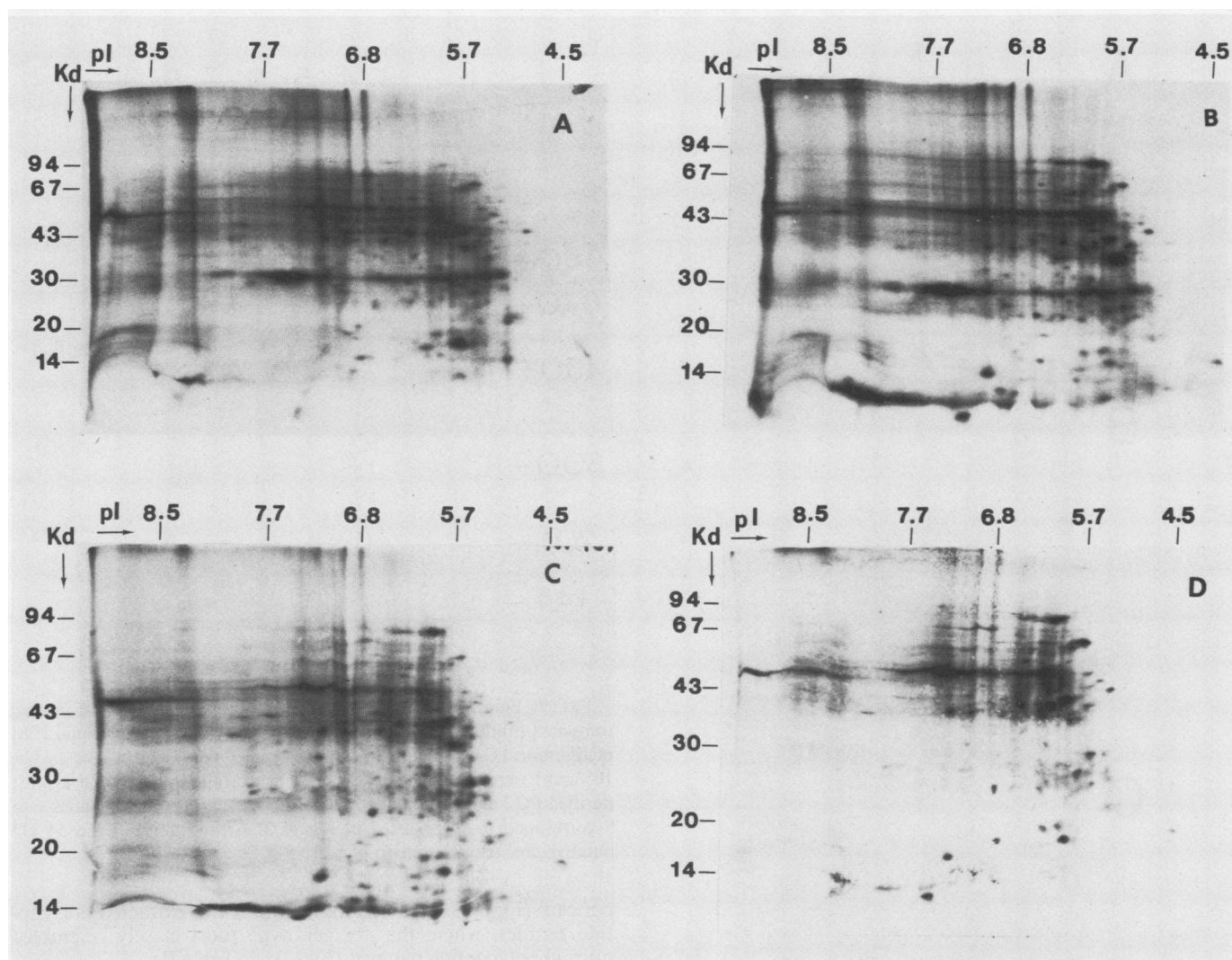
FIG. 5. Fluorograms of two-dimensional PAGE of [35S]methionine polypeptides translated *in vitro* from tomato pericarp poly(A)+ RNAs. A, MG and B, Tu from Rutgers; C, *Nr* 65 d; D, *rin* 65 d.

and *Nr* mutants of nearly isogenic background have not been performed. Evidence has been obtained based upon *in vitro* translations of polyribosomes, total RNAs, and poly(A)+ RNAs from various pericarp developmental stages (10, 22, 26, 27) that changes in gene expression are involved in the ripening process. However, no quantitative analyses of *in vitro* translation products in relation to the ripening process have been made. Furthermore, a discrepancy exists in the literature as to the size of a PG precursor as identified by immunoprecipitation of *in vitro* translation products (10, 24–26). This paper addresses some of the points noted above in an attempt to better understand the regulation of tomato fruit ripening as it relates to changes in mRNAs and proteins during this process.

Analysis of proteins present in tomato pericarp at various ripening stages revealed increases, decreases, and fluctuations in the levels of many polypeptides (Figs. 1, 2; Table II), thus suggesting that both synthesis and degradation of proteins are involved in the ripening process. Comparisons of polypeptides present in normal pericarp with those present in the *rin, nor,* and *Nr* mutants revealed several interesting features (Figs. 1, 2; Table II). First, several polypeptides present in normal pericarp exhibited reduced or nondetectable levels in mutant pericarp, especially the 44 kD polypeptide which comigrates with purified PG2. This polypeptide is greatly reduced in *Nr* pericarp and was

apparently absent in the *rin* and *nor* genotypes, which is in agreement with prior studies involving PG enzyme activity (5, 28, 29) or protein level as determined by immunoassay (5, 29). Second, several polypeptides detected in one or more of the mutant tissues were not observed in normal pericarp. All mutants contained polypeptides between 33 to 31 kD and another at 25 kD, both of which were either minor or absent in normal pericarp. Mizrahi *et al.* (18) also noted the presence of two low mol wt polypeptides in *rin* pericarp that were not present in normal ripening fruit, but since mol wts were not reported, a direct comparison to our results cannot be made. Third, the levels of many polypeptides decreased by 70 d after pollination, especially those of high mol wt. While degradation appears to be a function of chronological age rather than ripening status, the *rin* and *Nr* genotypes show this pattern to a greater extent than the normal and *nor* genotypes at similar ages.

Total RNA and poly(A)+ RNA levels obtained from normal pericarp peaked between the MG to Br stages (Table III), coinciding with the period during which several ripening-specific events, such as Chl degradation, carotenoid and PG biosynthesis, and increases in ethylene and CO_2 evolution are initiated (14, 23). Stimulation of protein synthesis around Br stage of tomato fruit ripening has been shown (8, 12). In addition, Speirs *et al.* (27) observed a peak of polyribosome content at this period in
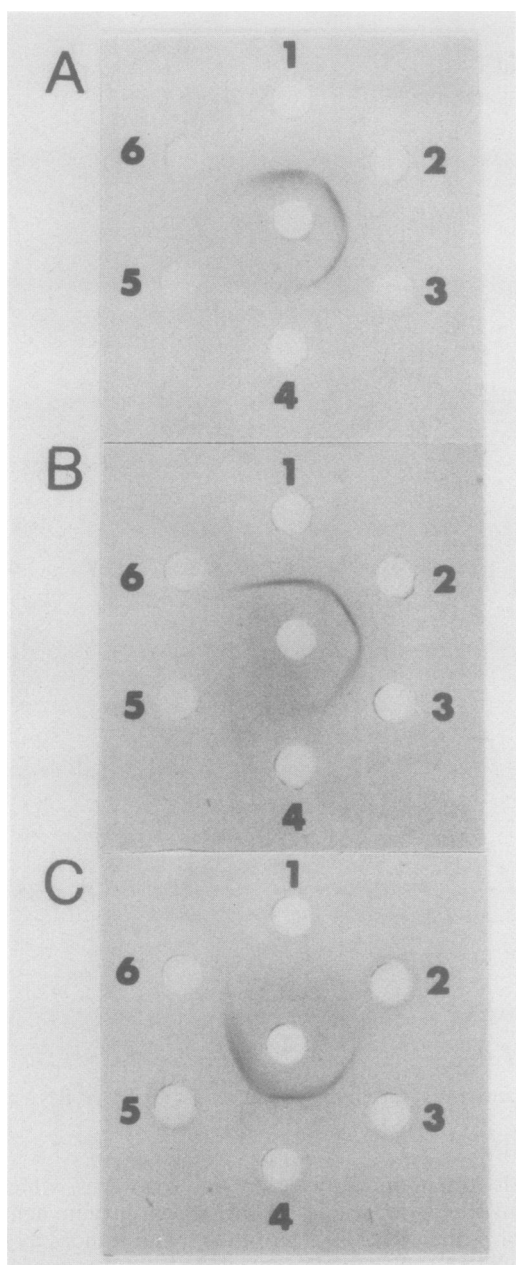
FIG. 6. Double immunodiffusion of PG protein and antibody. Various amounts of antigen (peripheral wells) were tested against 0.3 mg of total IgG (central wells). A and B, total antigen was applied as purified PG2 (A) or partially purified PG1 (B) in 0.15 M NaCl (pH 6.0) at (1) 5.00, (2) 2.50, (3) 1.25, (4) 0.63, (5) 0.32, and (6) 0 μg; C, 150 μg of total protein extracted from (1) IMG, (2) MG, (3) Br, (4) Tu, and (5) RR stages of normal pericarp was applied, with lane 6 containing 0.15 M NaCl only.

two other tomato fruit cultivars. Percent levels of poly(A)⁺ RNAs shown in this study (Table III) are in agreement with those reported by Mansson *et al.* (17).

Quantitative analyses of polypeptides translated in rabbit reticulocyte lysates from normal pericarp poly(A)⁺ RNAs (Fig. 3) showed increases in nine, decreases in two, and fluctuations in five polypeptides during ripening. Qualitative changes in some of these polypeptides have been reported earlier (10, 26, 27). Differences between *in vitro* translated polypeptides from normal *versus* mutant poly(A)⁺ RNAs were evident in all three mutants. The *rin* mutant yielded a profile similar to 70-d old normal (RR)
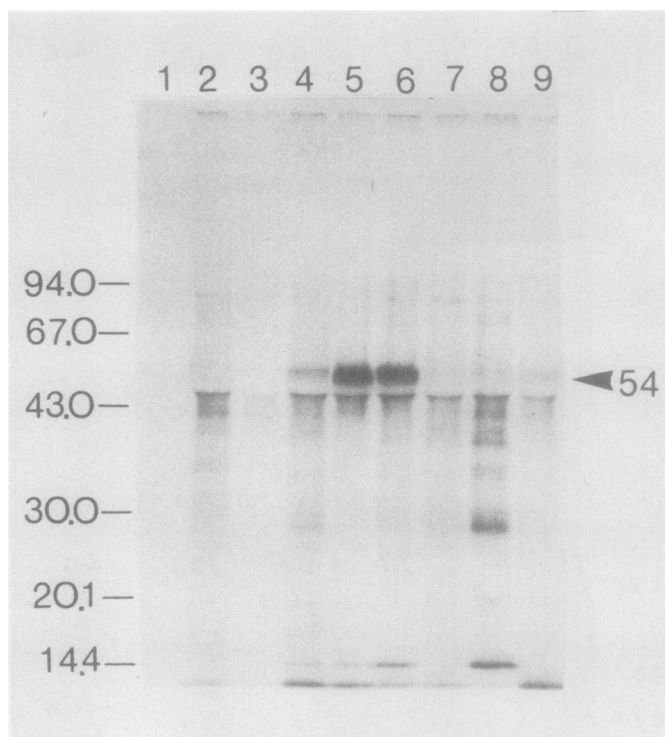

FIG. 7. Fluorogram of SDS-PAGE of immunoprecipitates of *in vitro* translated products from tomato pericarp poly(A)⁺ RNAs. Equal [³⁵S] methionine TCA-precipitable counts (5 × 10⁵ cpm) except lane 1 (3 × 10⁴ cpm) were incubated in the presence of antibodies raised against purified PG2 and immunocomplexes were electrophoresed. Lanes 1 to 9 correspond to those listed in Figure 3. Arrow indicates the 54 kD putative precursor of tomato pericarp PG.

pericarp (Figs. 3, 4; Table IV), in contrast to extracted polypeptide profiles, where the *nor* genotype most closely resembled normal polypeptide patterns (Figs. 1, 2; Table II).

From analyses of *in vitro* translation products (Figs. 3–5, 7) it seems that there are at least three classes of mRNAs present in ripening tomato fruits: those whose mRNA levels (a) do not show changes, (b) increase at or beyond the MG stage, and (c) decrease at or beyond the MG stage. Somewhat similar results have been reported by Speirs *et al.* (27) and Grierson *et al.* (10); however, their conclusions were not based upon quantitative analyses of translation profiles. Mansson *et al.* (17) reached similar conclusions from analysis of a cDNA library made from mRNAs isolated from ripe tomato fruit. They have shown by differential hybridization that among the population of ripe fruit mRNAs, about 2% are expressed predominately in ripe fruit, about 1% are expressed predominately in unripe fruit, and the remainder are expressed in both ripe and unripe fruits. Based upon our data, it seems that expression of mRNAs from classes 2 and 3 is pertubed in the *rin*, *nor*, and *Nr* mutants.

Immunoprecipitation of a PG precursor from polypeptides translated *in vitro* from ripe pericarp mRNAs has been reported (10, 24–26). Grierson *et al.* (10) and Slater *et al.* (26) reported immunoprecipitation of a 48 kD precursor from rabbit reticulocyte lysate translation products, while Sato *et al.* (24, 25) showed a 54 kD precursor immunoprecipitated from wheat germ system products. Using antibodies raised against purified PG2, we have immunoprecipitated a 54 kD precursor of PG after translation of poly(A)⁺ RNAs from Br, Tu, and RR stages as well as 65 d old *Nr* pericarp using rabbit reticulocyte lysate systems (Fig. 7). This is in agreement with the mol wt of PG precursor reported by Sato *et al.* (24, 25). The levels of 54 kD

precursor were highest from Tu and RR poly(A)$^+$ RNA translations but were not detected in IMG, MG, *rin*, or *nor* poly(A)$^+$ RNA translations. Since both PG mRNA and PG protein are either present or absent during the same stages of pericarp development, it suggests that the expression of PG is transcriptionally regulated.

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